REMARKS

Rejection of Claims 1-23 and 30-38 Under 35 U.S.C. § 103(a)

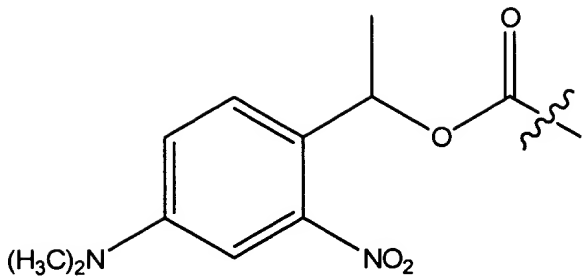
Claims 1-23 and 30-38 are rejected under 35 U.S.C. § 103(a) as being obvious over McGall *et al.* (U.S. Patent No. 5,412,087; hereinafter "the '087 Patent") in view of McGall *et al.* (WO 98/39348; hereinafter "the '348 Publication"). The Examiner states that the combination of the two references would have motivated one of ordinary skill in art to modify the teachings of the '087 Patent with the teaching of the '348 Publication to make compounds according to the present invention and to attach a molecule to a support in a method employing the protecting groups disclosed in the instant application.

In order to demonstrate that the present invention is not obvious in view of the cited references, Applicant encloses herewith a Declaration of Glenn McGall Under 37 C.F.R. § 1.132 (hereinafter "the Declaration").

In Sections 6, 7 and 9 of the Declaration, Dr. McGall explains that desirable photocleavable protecting groups typically have a high photospeed, create an exposed hydroxyl group on the protected molecule upon cleavage and do not generate undesirable side products that interfere with oligonucleotide synthesis. Moreover, as Dr. McGall states in Section 8 of the Declaration, photocleavable protecting groups for use in oligonucleotide synthesis are typically cleavable with 365 nm wavelength light. Shorter wavelength light causes damage to the oligonucleotides, particularly in terms of creating thymidine dimers, which inhibit binding to a complementary nucleic acid. Thus, cleavage at 365 nm is a highly desirable property for photocleavable protecting groups used in nucleotide synthesis.

However, according to Dr. McGall in Section 10 of the Declaration, not all "nitrobenzylic" protecting groups are suitable for oligonucleotide synthesis. Dr. McGall, referring to Table 1 in the '087 Patent, notes that many "nitrobenzylic" groups are not substantially cleaved with 365 nm light. Most of the groups, such as the DDZ group, listed therein are cleaved at shorter wavelengths, which is problematic if the groups are to be used in preparing oligonucleotides.

In addition, in Section 11, Dr. McGall states that the "nitrobenzylic" group represented by the following structure is not at all cleavable with 365 nm light:



In view of this evidence, Dr. McGall states that the '087 Patent does not render every desirable photocleavable protecting group obvious. The '348 Publication is related to bicyclic and polycyclic groups and therefore does not remedy the deficiencies of the '087 Patent.

Because many protecting groups are not cleavable with 365 nm light, as demonstrated above, it is unexpected that each of the groups recited in the pending claims are cleavable with 365 nm light. One of ordinary skill in the art would have had to perform extensive experimentation in order to prepare suitable compounds, such as those recited in the instant claims. It is emphasized that the ability to be cleaved by 365 nm light (and not a shorter wavelength) is important for protecting groups used in oligonucleotide synthesis.

Because each of the protecting groups recited in the claims have at least one property that is unexpected in view of the cited references, Claims 1-23 and 30-38 are not obvious over the '087 Patent in view of the '348 Publication. Many of the protecting groups exemplified in the cited references are not substantially cleaved by 365 nm light. In contrast, each of the claimed photocleavable protecting groups has this desirable property. Reconsideration and withdrawal of the rejection are respectfully requested.

CONCLUSION

In view of the above remarks and the Declaration submitted herewith, it is believed that all claims are in condition for allowance, and it is respectfully requested that the application be

passed to issue. If the Examiner feels that a telephone conference would expedite prosecution of this case, the Examiner is invited to call the undersigned.

Respectfully submitted,

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EDUCATION

• 1986	Ph.D.	Organic Chemistry	University of Toronto
• 1981	B.Sc.	Chemistry and Biochemistry	University of Toronto

PROFESSIONAL EXPERIENCE

Affymetrix, Inc. Santa Clara, CA. Chemistry Dept., Manager (1993-98); Director (1998-99); Chemistry and Process Development, Sr. Director. (1999-present).

- Responsible for establishing and directing technical programs aimed at developing and implementing processes for manufacturing high-density oligonucleotide arrays, and for the modification and labeling of nucleic acids for detection of hybridization on DNA probe arrays.
- Supervised chemistry research and process development departments comprised of PhD, BS/ MS-level scientists and engineers, and technicians. Managed departmental budgets.
- Participated in the set up and ongoing consulting and troubleshooting of manufacturing facility for high-density DNA arrays, specifying and optimizing procedures for synthesis and substrate preparation, as well as development of reagents and analytical QC methods.
- Supervised & assisted with internal manufacturing of photolabile reagents for chip manufacturing.
- Responsible for selecting and qualifying outside vendors to supply reagents for manufacturing. Generated production protocols and analytical specifications for phosphoramidite and biotinylated nucleotide reagents, and provided ongoing technical support to external vendors and internal QC departments.
- Established and managed contracts with outside synthesis and analytical laboratories to provide custom services in support of internal R&D projects.
- Developed new photolabile phosphoramidite chemistries for photolithographic array synthesis and for the introduction of modified nucleotides, linkers and fluorescent molecules into probe arrays.
- Established a program to enhance the hybridization characteristics of nucleic acid probe arrays by incorporating modified nucleotides and nucleotide analogs into the array (synthetically), and into target nucleic acids (enzymatically).
- Established a program to develop novel nucleotide analogs for the labeling and detection of nucleic acids.
- Responsible for managing external corporate and academic research collaborations.
- Very active in the IP area, generating or contributing to numerous patent applications, as well as initiating and participating in a successful patent opposition at the European Patent Office.
- Author/co-author on 12 scientific publications.

Affymetrix, Inc. Santa Clara, CA. Staff Scientist. (1991-1993)

- Led the development of chemical processes for fabricating DNA probe arrays using light-directed immobilization and *in situ* synthesis.
- Participated in the development of DNA array manufacturing methods and the associated assay and detection systems for use in sequencing applications.
- Supervised in-house production of photoactivatable nucleoside & linker phosphoramidites for array manufacturing.
- Developed methods for the analysis of DNA synthesis efficiency on planar supports.

Syva Company, Palo Alto, CA. Senior Chemist, Research Department. (1988-1991)

- Development of new chemistries for novel antibody- and DNA probe-based diagnostic assays.
- Synthesis of haptens, conjugation to enzymes and antibodies.
- Oligonucleotide synthesis and labeling with fluorescent, chemiluminescent molecules.

M.I.T. Department of Chemistry, Cambridge, MA; Postdoctoral Research Fellow (1986-1988)

- Mechanistic study of DNA degradation by antitumor drugs.
- Synthesis of nucleosides, nucleotides and polynucleotides labeled with stable- and radio-isotopes.
- Isolation and quantitative analysis of ng quantities of reaction products using radioactivity and GC-MS.

EXHIBIT
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- Published 4 scientific papers in refereed journals, and one invited review.

University of Toronto, Department of Chemistry, Toronto, Canada; PhD Candidate. (1981-1985)

- Kinetic and mechanistic study of alkoxyphosphorane hydrolysis, a model for biochemical phosphoryl transfer reactions. Published 8 scientific papers in refereed journals.

ACADEMIC FELLOWSHIPS and AWARDS

- 1984-1985 NSERC Postgraduate Scholarship
- 1981-1983 University of Toronto Postgraduate Scholarship
- 1981-1984 University of Toronto Lash-Miller Postgraduate Award
- 1980 Canadian Institute of Chemistry Silver Medal for Undergraduate Study

PUBLICATIONS

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Novel nucleotide analogs for end labeling DNA. Barone A, Chen C, Combs D, Li H, McGall G. In Preparation.
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Patents

Issued patents and published applications follow. Additional cases currently pending.

Issued US patents

1. 6,773,888 Photoactivatable silane compounds and methods for their synthesis and use
2. 6,770,436 Chemical amplification for the synthesis of patterned arrays
3. 6,743,882 Functionalized silicon compounds and methods for their synthesis and use
4. 6,706,875 Substrate preparation process
5. 6,667,394 Printing oligonucleotide arrays
6. 6,632,605 Hybridization assays on oligonucleotide arrays
7. 6,596,856 Nucleic acid labeling compounds
8. 6,576,425 Methods for testing oligonucleotide arrays
9. 6,566,515 Photocleavable protecting groups and methods for their use
10. 6,486,287 Functionalized silicon compounds and methods for their synthesis and use
11. 6,486,286 Use of hexahydrolupulones as antibacterial agents
12. 6,429,275 Functionalized silicon compounds and methods for their synthesis and use
13. 6,410,675 Functionalized silicon compounds and methods for their synthesis and use
14. 6,307,042 Substrate preparation process
15. 6,262,216 Functionalized silicon compounds and methods for their synthesis and use
16. 6,239,273 Printing molecular library arrays
17. 6,238,862 Methods for testing oligonucleotide arrays
18. 6,156,501 Arrays of modified nucleic acid probes and methods of use
19. 6,150,147 Biological array fabrication methods with reduction of static charge
20. 6,147,205 Photocleavable protecting groups and methods for their use
21. 6,083,697 Chemical amplification for the synthesis of patterned arrays
22. 6,045,996 Hybridization assays on oligonucleotide arrays
23. 6,022,963 Synthesis of oligonucleotide arrays using photocleavable protecting groups

24. 5,959,098 Substrate preparation process
25. 5,919,523 Derivatization of solid supports and methods for oligomer synthesis
26. 5,843,655 Methods for testing oligonucleotide arrays
27. 5,831,070 Printing oligonucleotide arrays using deprotection agents solely in the vapor phase
28. 5,658,734 Process for synthesizing chemical compounds
29. 5,599,695 Printing molecular library arrays using deprotection agents solely in the vapor phase
30. 5,412,087 Spatially-addressable immobilization of oligonucleotides and other biological polymers on surfaces

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1. 20040106728 Reagents and methods for solid phase synthesis and display
2. 20040105932 Substrate preparation process
3. 20040101892 Nucleic acid labeling compounds
4. 20040092396 Porous silica substrates for polymer synthesis and assays
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10. 20030232979 Nucleic acid labeling compounds
11. 20030232361 Nucleic acid array preparation using purified phosphoramidites
12. 20030194715 Photoactivatable silane compounds and methods for their synthesis and use
13. 20030180757 Nucleic acid labeling compounds
14. 20030175409 Printing molecular library arrays
15. 20030144499 Photocleavable protecting groups and methods for their use
16. 20030077650 Functionalized silicon compounds and methods for their synthesis and use
17. 20030064364 Nucleic acid analysis techniques
18. 20030040618 Photocleavable protecting groups
19. 20030017451 Methods for detecting transcripts
20. 20020182625 Nucleic acid labeling compounds
21. 20020165372 Nucleic acid labeling compounds
22. 20020147319 Printing oligonucleotide arrays
23. 20020022721 Methods of array synthesis
24. 20020009729 Methods for testing oligonucleotide arrays
25. 20010049108 Methods for reducing non-specific binding to an oligonucleotide array
26. 20010044531 Nucleic acid labeling compounds
27. 20010027187 Functionalized silicon compounds and methods for their synthesis and use
28. 20010021506 Functionalized silicon compounds and methods for their synthesis and use
29. 20010018514 Nucleic Acid Labeling Compounds
30. 20010014453 Functionalized silicon compounds and methods for their synthesis and use

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1. WO 2004 007751 Nucleic acid labeling methods and compounds for use with microarray hybridization.
2. WO 2002 077283 Methods for gene expression profiling and single nucleotide polymorphism detection using microarrays.
3. WO 2002 020150 Preparation of nucleotides using photo-cleavable protecting groups.
4. EP 2001 728520 Printing molecular library arrays.
5. EP 2001 081163 Macromolecular arrays on polymeric brushes and methods for preparing the same.
6. WO 2000 061282 Porous silica substrates for polymers synthesis and assays.
7. WO 2000 021967 Functionalized silicon compounds their synthesis and use.
8. WO 2000 006771 Nucleotide analogs and their use in labeling nucleic acids for hybridization assays.
9. EP 1999 967217 Reagents and methods for solid phase synthesis of oligonucleotide and peptides.
10. WO 1999 954509 Methods for reducing non-specific binding to a nucleic acid probe array by controlled modification of probes or immobilizing surfaces.
11. WO 1999 940105 Array fabrication methods
12. WO 1999 839348 Photocleavable protecting groups and methods for their use.
13. WO 1997 739151 Photolabile Polymer Array Synthesis Methods.
14. WO 1997 727317 Nucleic Acid Analysis Techniques.
15. WO 1997 743450 Hybridization buffers and media improving the signal-to-noise ratio for assays on oligonucleotide arrays.

16. EP 1996 742287 Oligonucleotide analog probe arrays immobilized on solid substrates target nucleic acid analogs and probe-target improved hybridization
17. EP 1993 549107 Method for amplifying single-strand target polynucleotide using modified extender probe.

Carcinogenesis vol.18 no.4 pp.811–816, 1997

Wavelength dependence of oxidative DNA damage induced by UV and visible light

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DNA damage induced by UV radiation and visible light (290–500 nm) in AS52 Chinese hamster cells was analysed by an alkaline elution assay with specific repair endonucleases. Cells were exposed to extensively filtered monochrome or broad-band radiation. Between 290 and 315 nm, the ratio of base modifications sensitive to Fpg protein (i.e. 8-hydroxyguanine and formamidopyrimidines) and T4 endonuclease V (i.e. cyclobutane pyrimidine dimers) was constant (~1:200), indicating that the direct excitation of DNA is responsible for both types of damage in this range of the spectrum. While the yield of pyrimidine dimers per unit dose continued to decrease exponentially beyond 315 nm, the yield of Fpg-sensitive modifications increased to a second maximum between 400 and 450 nm. The damage spectrum in this wavelength range consisted of only a few other modifications (strand breaks, abasic sites and pyrimidine modifications sensitive to endonuclease III) and is attributed to endogenous photosensitizers that give rise to oxidative DNA damage via singlet oxygen and/or type I reactions. The generation of Fpg-sensitive modifications by visible light was not linear with dose but followed a saturation curve. It is calculated that the exposure of the cells to low doses of solar radiation results in the formation of cyclobutane pyrimidine dimers and Fpg-sensitive modifications in a ratio of 10:1.

Introduction

Solar radiation can give rise to cellular DNA damage by (i) direct excitation of DNA and (ii) indirect mechanism(s) that involve the excitation of other cellular chromophores (endogenous photosensitizers) (1,2). The direct excitation of DNA generates predominantly cyclobutane pyrimidine dimers and (6–4) photoproducts, which are without doubt of principal importance for the cytotoxic, mutagenic and carcinogenic effects of short-wave UV radiation (UVC, UVB) (3–7). On the other hand, indirect mechanisms must be responsible for DNA damage and genotoxic effects observed at longer wavelengths (UVA, visible light), at which DNA absorbs only weakly or not at all (8,9). In this range of the spectrum, various oxidative DNA modifications such as 8-hydroxyguanine (8-oxoG*), strand breaks, sites of base loss and DNA-protein crosslinks are generated (10–12). The mutagenic potential of some of these lesions, e.g. 8-oxoG, has been well established (13,14). Therefore, it is not surprising that the wavelength dependence of mutation induction by UV does not correlate with that of pyrimidine dimer formation (15) and that the action spectra obtained for photocarcinogenesis (16,17) indicate (or are at least compatible with) a role of non-dimer modifications in skin cancer induction.

The mechanisms involved in the generation of oxidative DNA modifications, in particular the specific absorbing chromophore(s) and the reactive species involved, remain to be established. In principle, an excited endogenous photosensitizer molecule could react with DNA without further intermediates (type I reaction) or via singlet oxygen ($^1O_2$) (type II reaction) (Figure 1). Alternatively, hydroxyl radicals ($^{\cdot}OH$) — generated via superoxide (O_2^-) and a Fenton reaction — could be involved. Yet another possibility is a release of Ca^{2+} from intracellular stores which could activate cellular nucleases and thus induce DNA damage (18,19).

The ratio of pyrimidine dimers and oxidative DNA modifications at various wavelengths depends on the relative contribution of direct and indirect mechanisms and is of major interest for the estimation of the mutagenic risk associated with solar irradiation and the impact of spectral changes caused by ozone layer depletion. Here, we report a quantification of cyclobutane pyrimidine dimers and various types of oxidative modifications induced in cultured cells by wavelengths between 290 and 500 nm. The lesions were quantified by means of several repair endonucleases (Table I) and an alkaline elution technique (28).

The results indicate that the generation of oxidative DNA base modifications by solar radiation has a second maximum between 400 and 450 nm due to the excitation of (unidentified) endogenous photosensitizers and that in cells exposed to sunlight these indirectly induced base modifications account for ~10% of the total endonuclease-sensitive base damage.

Materials and methods

Cells and repair endonucleases

AS52 Chinese hamster ovary cells, which carry the bacterial *gpt* gene for analysis of mutations (29), were obtained from W.J.Caspary, Research Triangle

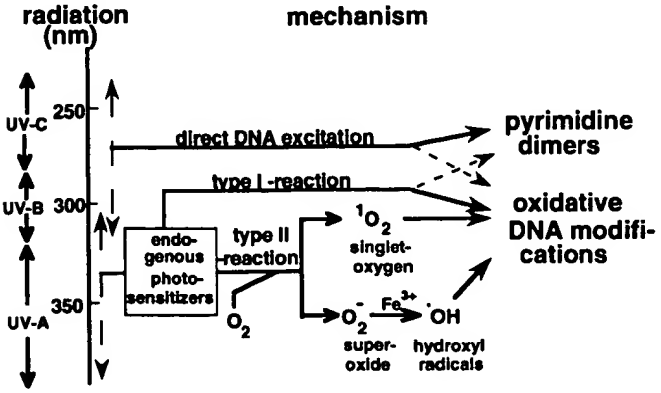

Fig. 1. Direct and indirect mechanisms for the induction of pyrimidine dimers and oxidative DNA modifications by UV and visible light.

*Abbreviations: 8-oxoG, 8-hydroxyguanine; Fpg protein, formamidopyrimidine-DNA glycosylase; MMS, methyl methanesulfonate; Fapy-A, 4,6-diamino-5-formamidopyrimidine.

Table I. Recognition of DNA modifications by repair endonucleases used in this study

Repair endonuclease	Recognition spectrum[a] Sites of base loss[b]	Base modifications
Fpg protein[c]	+	8-oxoG[d]; Fapy[e]
Endonuclease III[c]	+	5,6-dihydropyrimidines; hyd[f]
T4 endonuclease V	+	Py <> Py[g,h]
Exonuclease III	+	—

[a]See refs 20–24.
[b]For the recognition of sites of base loss oxidized in the 1′ or 4′ position, see ref. 25.
[c]5-Hydroxycytosine and 5-hydroxyuracil have recently been described as new substrates of both Fpg protein and endonuclease III (26).
[d]7,8-Dihydro-8-oxoguanine (8-hydroxyguanine).
[e]Formamidopyrimidines (imidazole ring-opened purines).
[f]5-Hydroxy-5-methylhydantoin and other ring-contracted and fragmented pyrimidines.
[g]Cyclobutane pyrimidine photodimers.
[h]At 100-fold higher concentration, 4,6-diamino-5-formamidopyrimidine (Fapy-A) was also found to be a substrate of T4 endonuclease V (27).

Park, USA, and cultured in Ham's F12 medium containing 5% fetal calf serum, penicillin (100 units/ml) and streptomycin (100 μg/ml).

Formamidopyrimidine-DNA glycosylase (Fpg protein) (30) and endonuclease III from *E.coli* were kindly provided by S.Boiteux, Fonteney aux Roses, France. T4 endonuclease V was partially purified by the method described by Nakabeppu *et al.* (31) from the *E.coli* strain A 32480 (*uvrA, recA*, F′lac IQ1). The strain carries the plasmid ptac-denV (kindly provided by L.Mullenders, Leiden, Netherlands). Expression of T4 endonuclease V is induced with isopropyl-β-D-thiogalactopyranoside. Exonuclease III was purchased from Boehringer Mannheim, Germany. All repair endonucleases were tested under cell-free conditions for their incision at reference modifications (i.e. thymine glycols induced by OsO_4, AP sites by low pH and 8-oxoG by methylene blue plus light) to ensure that the correct substrate modifications are fully recognized and no incision at non-substrate modifications takes place (32).

Light sources and irradiation conditions

Irradiation with UV and visible light was carried out at 0°C in PBS buffer (137 mM NaCl, 2.7 mM KCl, 8.3 mM NaH_2PO_4, 1.5 mM KH_2PO_4, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.4) either with a broad-spectrum UVB source (Philips TL20W/12RS; maximum emission at 306 nm) filtered by the polystyrene of the culture flasks (~1.3 W/m^2), or with a 1000 W xenon arc lamp (976C-0010, Hanovia, USA) with a grid monochromator (bandwidth 8 nm) and/or cut-off filters (~4 W/m^2/nm). For irradiation with the xenon lamp, the cells were suspended in a quartz tube (3×10^6 cells in 3 ml PBS buffer). The transmission of the cell suspension was >50% at all wavelengths between 290 and 600 nm. Cut-off filters (Schott, Mainz, Germany; 3 mm; two in line) with half-maximal absorptions at 335, 360, 400, 420, 455, 475 and 495 nm were used to define wavelength intervals in the determination of the action spectra. The dosimetry was performed with a calibrated dosimeter (Krochmann GmbH, Berlin, Germany) equipped with a GaP-photoelement.

Quantification of endonuclease-sensitive modifications

Determination of endonuclease-sensitive modifications was carried out by means of an alkaline elution assay, in which the cellular DNA was incubated with one of the repair endonucleases [Fpg protein (1 μg/ml), endonuclease III (10 ng/ml), T4 endonuclease V (30 ng/ml) or exonuclease III (0.5 μg/ml)] immediately after cell lysis, as described previously (28,33). To quantify DNA single-strand breaks, the incubation was carried out without endonuclease. The alkaline elution followed the method of Kohn *et al.* (34) with modifications (28,35). For the quantitative evaluation, the slope of an elution curve obtained with γ-irradiated cells was used for calibration (6 Gy = 1 ssb/10^6 bp). The slopes observed with untreated control cells were subtracted. The numbers of modifications incised by the repair endonucleases were obtained by subtraction of the number of single-strand breaks observed without endonuclease treatment.

Quantification of cyclobutane pyrimidine dimers by monoclonal antibodies

A monoclonal mouse H3 antibody (36) was used to quantify cyclobutane pyrimidine dimers in an immuno dot-blot assay as described previously (37). The antibody recognizes TT and 5′-TC cyclobutane dimers, which account for ~70% of all cyclobutane dimers induced by UVB (38), but not (6–4) photoproducts.

Table II. Comparison of quantifications of cyclobutane pyrimidine dimers by alkaline elution and immunoassay

Inducing radiation	Dose applied (kJ/m^2)		Modifications detected per kJ/m^2 per 10^6 bp	
	Alk. elution	Immunoassay	Alk. elution[a]	Immunoassay[b]
300 nm	0.005	0.36–2.9	89±10	101±8
310 nm	0.2	17–34	3.7±0.5	3.2±0.2
320 nm	3	35–70	0.17±0.05	0.16±0.2
UVB	0.0066	0.79–2.4	77±13	139±39

[a]Sites sensitive to T4 endonuclease V determined by alkaline elution.
[b]Sites detected in an immuno dot blot assay using the monoclonal mouse H3 antibody.

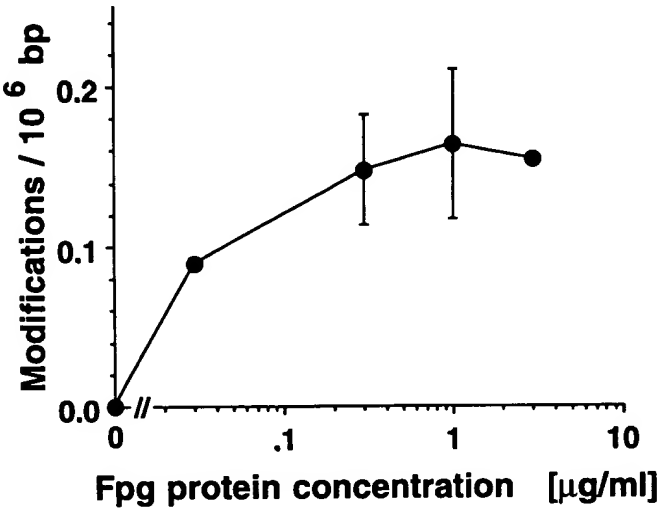

Fig. 2. Recognition by various concentrations of Fpg protein of DNA modifications induced by visible light (>400 nm). Data are obtained from 1 or 3 independent experiments (± SD).

Results

Validation of the assay system

The reliability of the alkaline elution assay when used in combination with repair endonucleases to quantify endonuclease-sensitive DNA modifications was verified in three types of experiment. Firstly, cyclobutane pyrimidine dimers induced by UVB in AS52 (Chinese hamster ovary) cells were quantified both with T4 endonuclease V in combination with the alkaline elution technique and with an immunoassay (see Materials and methods). The two independent assay systems gave similar results (Table II). Secondly, the enzyme concentration dependence for the recognition by Fpg protein of visible light-induced oxidative modifications was determined. As shown in Figure 2, the number of sites detected by the enzyme was saturated at 1 μg/ml Fpg protein. This concentration is similar to that required for a full recognition of base modifications induced under cell-free conditions by methylene blue plus light (32). Thirdly, the recognition by the various enzymes of sites of base loss induced by methyl methanesulfonate (MMS) was analysed. T4 endonuclease V, endonuclease III and exonuclease III detected the same number of modications (data not shown), in agreement with the fact that sites of base loss are substrates for all three enzymes (Table I).

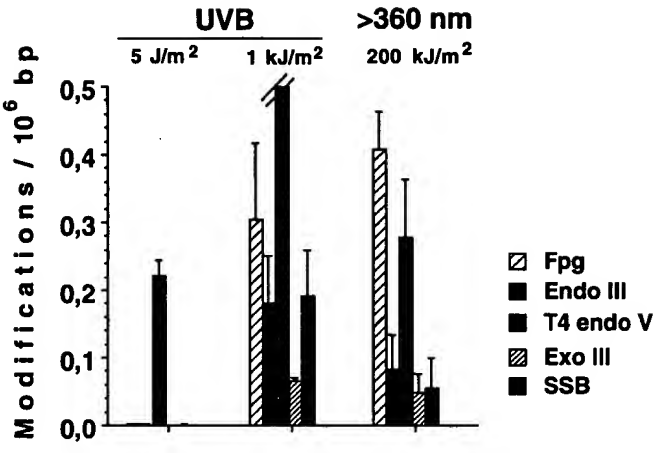

Fig. 3. DNA damage profiles induced in AS52 cells by broad spectrum UVB (Philips TL20W/12RS filtered by polystyrene culture flasks) and by wavelengths >360 nm (xenon arc lamp). Columns indicate the numbers of various endonuclease-sensitive modifications (first to fourth column) and the number of single-strand breaks (fifth column). Data represent the means of 3–7 independent experiments (± SD).

Comparison of DNA damage profiles

The numbers of various endonuclease-sensitive modifications (see Table I) induced in AS52 by two spectral ranges of radiation are shown in Figure 3 in the form of DNA damage profiles. After broad spectrum UVB irradiation, 99% of all modifications detected were pyrimidine dimers sensitive to T4 endonuclease V. The sites sensitive to endonuclease III and Fpg protein were mostly base modifications, since the number of AP sites detected by exonuclease III was even lower. After irradiation with wavelengths above 360 nm from a xenon lamp, T4 endonuclease V-sensitive base modifications were still detectable (Figure 3). Since an additional cut-off filter at 360 nm had no effect on the number of modifications generated per dose unit (data not shown), the pyrimidine dimers cannot be attributed to low amounts of radiation emitted below 360 nm. Base modifications sensitive to Fpg protein were formed in a yield similar to pyrimidine dimers, while sites of base loss (sensitive to exonuclease III), single-strand breaks and oxidative pyrimidine modifications (sensitive to endonuclease III) were much less frequent.

Effects of cut-off filters on the damage profiles induced by UV radiation from a monochromator

Damage profiles observed in AS52 cells exposed to 310, 320 and 330 nm radiation from a monochromator (8 nm band width) are depicted in Figure 4. For all three wavelengths, the effects of one or two additional cut-off filters (with half-maximal absorption at 295, 305 and 320 nm, respectively) are also shown. The data indicate that the elimination of wavelengths well below the nominal settings of the monochromator had no effect on the generation of non-dimer modifications (single-strand breaks, modifications sensitive to endonuclease III and Fpg protein) and of pyrimidine dimers induced at 310 nm. However, the cut-off filters significantly reduced the number of pyrimidine dimers per dose unit induced with monochromator settings at 320 and 330 nm. A second cut-off filter of the same type had no further effect in any case. Therefore, a significant part—but not all—of the pyrimidine dimers induced at 320 nm and 330 nm can be attributed to the low percentage of radiation below 305 and 320 nm, respectively. In contrast, this low dose of radiation with shorter wavelengths does not contribute significantly to the generation of the Fpg-sensitive modifications. Therefore, different mechanisms must be responsible for the two types of damage.

Dose dependence

The number of pyrimidine dimers sensitive to T4 endonuclease V increased linearly with dose in the dose range analysed (i.e., in which 0.05–1 modifications per 10^6 bp were induced). In contrast, the number of Fpg-sensitive modifications induced in the visible range of the spectrum followed a saturation curve, i.e. the extent of damage did not exceed 0.3 modifications per 10^6 bp (Figure 5).

Action spectra

The generation of single-strand breaks and modifications sensitive to T4 endonuclease V and Fpg protein in AS52 cells at various wavelengths calculated for a given dose (20 kJ/m^2) is shown in Figure 6 in the form of action spectra. In all experiments, the actual doses applied were chosen to yield <1 modification per 10^6 bp. Up to the doses applied, all types of modifications were shown to increase linearly with dose (see above). A monochromator in line with an additional cut-off filter (with half-maximal absorption at wavelengths 10–15 nm below the nominal settings of the monochromator) was used in the range between 290 and 330 nm. For longer wavelengths, no monochromator could be used and the differences observed in experiments with cut-off filters (two in line) at two different wavelengths were determined. These were used to calculate the number of modifications per unit dose for the arithmetic mean of the two cut-off wavelengths.

The action spectra indicate that numbers of single-strand breaks, Fpg-sensitive modifications and T4 endonuclease-sensitive sites (cyclobutane pyrimidine dimers) decreased in parallel between 290 and ~315 nm. The T4 endonuclease-sensitive sites continued to decrease exponentially at longer wavelengths, but were still detectable up to 380 nm. In contrast, the number of Fpg-sensitive modifications per unit dose had a minimum around 340 nm and rose to a long-wave maximum between 400 and 450 nm. Single-strand breaks were detectable in all wavelength ranges. The yield was similar to that of Fpg-sensitive modifications in the UVB range, but was much lower than that of Fpg-sensitive modifications in the visible range.

Discussion

The results presented above provide quantitative information on the ratio of pyrimidine dimers and oxidative DNA base modifications induced in mammalian cells at various wavelengths of solar radiation. In addition, they allow mechanistic conclusions and an estimation of the contribution of oxidative base modification to the mutagenicity of sunlight.

The wavelength dependence of the induction of pyrimidine dimers (T4 endonuclease sensitive sites) in AS52 cells (Figure 6) closely resembles that reported previously for other types of cultured cells (15,39). It is also similar to that observed for the induction of both cyclobutane pyrimidine dimers and (6–4) photodimers in isolated DNA (40). The induction of dimers in the UVA range of the spectrum therefore can be attributed to the (very weak) end absorption of DNA and need not be mediated by other cellular chromophores, which in principle could generate cyclobutane pyrimidine dimers by triplet-triplet energy transfer (33,41). The recently reported (27) weak recognition by T4 endonuclease V of 4,6-diamino-

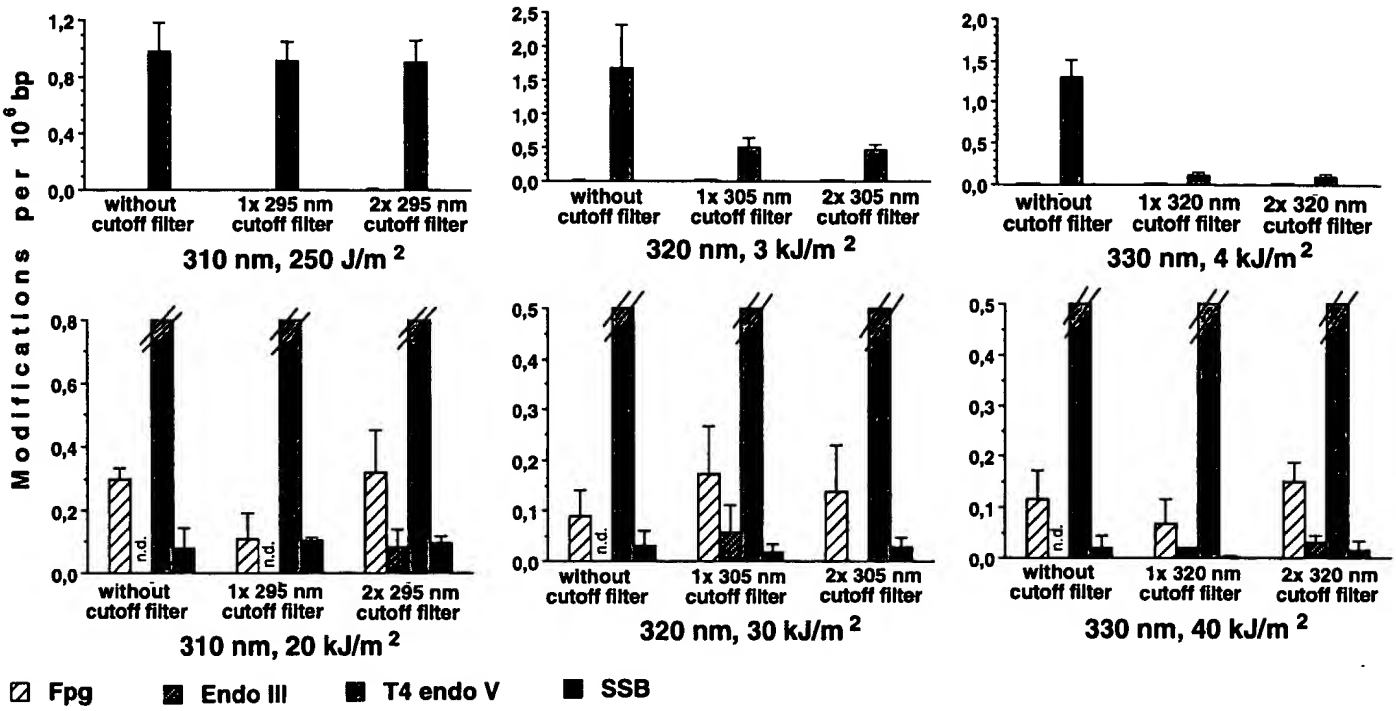

Fig. 4. Effects of one or two cut-off filters on the DNA damage profiles induced in AS52 cells by radiation from a monochromator-coupled xenon lamp (band width 8 nm). Data represent the means of 3–6 independent experiments (± SD).

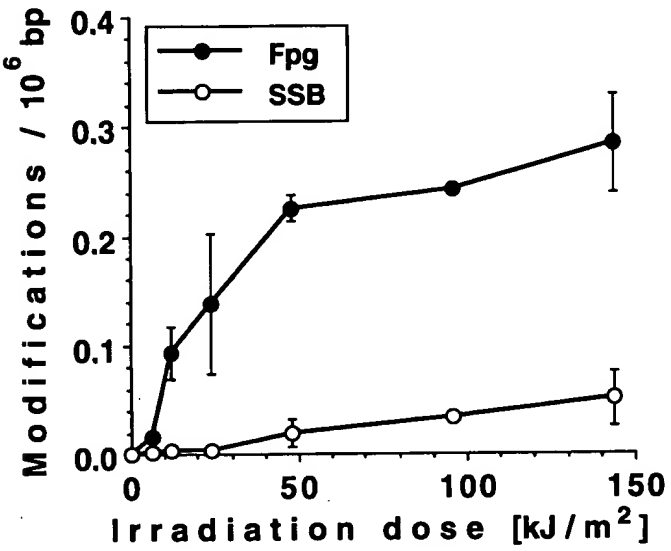

Fig. 5. Dose dependence of the induction of Fpg-sensitive modifications (●) and single-strand breaks (○) in AS52 cells by visible light (>400 nm) from a xenon arc lamp. Dose values given on the abscissa refer to the range between 400 and 500 nm. Data represent the means of 3–5 independent experiments (± SD).

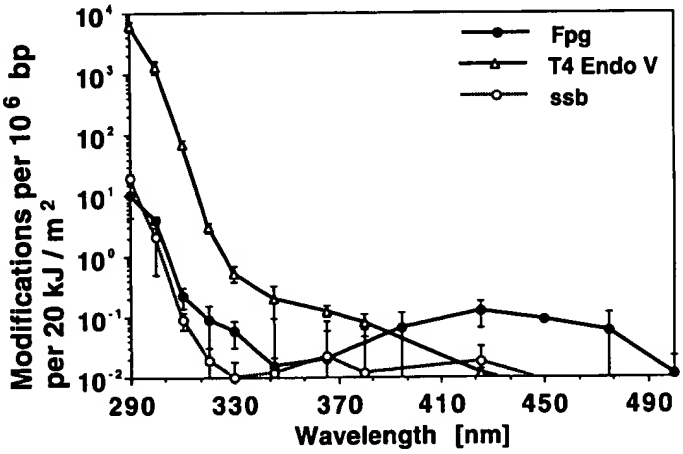

Fig. 6. Wavelength dependence of the induction of Fpg-sensitive modifications (●) T4 endonuclease V-sensitive modifications (△) and single-strand breaks (○) in AS52 cells calculated for a dose of 20 kJ/m^2. Between 290–330 nm, radiation passed through a grid monochromator (band width 8 nm) and an additional cut-off filter with half-maximal absorption 5–15 nm below the nominal wavelength of the monochromator. Between 340 and 500 nm, only cut-off filters were used (see description in the text). In the dose range applied for the determinations, all types of modifications were shown to increase linear with dose. Data represent the means of 3–7 independent experiments (± SD).

5-formamidopyrimidine (Fapy-A) cannot account for the T4 endonuclease-sensitive sites induced by UVA since (i) the enzyme concentrations required to for a (very incomplete) excision of Fapy-A were >300-fold higher than those used in this study and (ii) Fapy-A is a good substrate for Fpg protein and there is only a narrow wavelength range (around 380 nm) in which there is a significant recognition by T4 endonuclease V in the presence of an (at least) similarly high number of Fpg-senitive sites.

In contrast to pyrimidine dimers, base modifications sensitive to Fpg protein are induced in the whole wavelength range between 290 and 470 nm (Figure 6). Within the UVB range (290–315 nm), the ratio of Fpg-sensitive modifications and pyrimidine dimers is nearly constant and follows the DNA photon absorption. Therefore, these Fpg-sensitive modifications arise (mostly) from the direct excitation of DNA. Chemically, they may consist of both 8-oxoG and formamidopyrimidines. Both types of modification are established substrates of Fpg protein (Table I). 8-OxoG has

been detected in UVB-irradiated mammalian cells (42). Formamidopyrimidines have been identified in DNA irradiated under cell-free conditions with UVB (43). Since according to a recent report, 5-hydroxycytosine and 5-hydroxyuracil are additional substrates of both Fpg protein and endonuclease III (26), the presence of these lesions (up to the extent of endonuclease III-sensitive base modifications) can also not be excluded. Only some of the Fpg-sensitive modifications are sites of base loss, since the number of sites detected by exonuclease III, which recognizes sites of base loss only (Table I), is much lower.

Beyond 350 nm, the yield of Fpg-sensitive base modifications increases to a relative maximum between 400 and 450 nm (Figure 6). In this range of the spectrum, no or very few other modifications (single-strand breaks, AP sites, pyrimidine modifications sensitive to endonuclease III) are generated (Figures 3 and 6), as observed previously for another cell line (44). The damage profile thereby resembles that induced by singlet oxygen or type I photosensitizers under cell-free conditions (35), in support of the assumption that endogeneous photosensitizers are responsible and modify the DNA either directly (type I reaction) or via singlet oxygen (type II reaction), but not via hydroxyl radicals. Since according to cell-free studies (45), only a few formamidopyrimidines are generated by these mechanisms, 8-oxoG is likely to be the principal modification produced in this wavelength range. The formation of 8-oxoG in cells treated with riboflavin and visible light has been reported (46).

Like Fpg-sensitive modifications, single-strand breaks were induced in all ranges of the spectrum, in agreement with earlier observations that they too can be generated by both direct and indirect mechanisms (11). In addition, they may (in part) arise from sites of base loss, in particular oxidized sites of base loss, which are converted into strand breaks under the alkaline elution conditions.

The generation of base modifications sensitive to endonuclease III is largely restricted to UVB (Figure 3). According to earlier studies, these endonuclease III-sensitive modifications are cytosine hydrates and other oxidized pyrimidines formed in consequence of a direct excitation of DNA in a yield of ~1% of that of cyclobutane pyrimidine dimers (47–49).

The action spectra shown in Figure 6 in combination with published solar emission spectra (50,51) make it possible to calculate that solar radiation induces Fpg-sensitive base modifications, most of which presumably are 8-oxoG, in a yield of up to 10% of that of pyrimidine dimers. Exposures of keratinocytes for a few minutes to direct sunlight indeed confirmed that Fpg-sensitive modifications and T4 endonuclease-V-sensitive modifications are generated in a ratio of 1:10 (data not shown). At the *gpt* locus of AS52 cells, the number of Fpg-sensitive modifications induced by various (exogenous) photosensitizers that gave rise to (or at least was associated with) a given number of mutations was similar to the number of T4 endonuclease-V-sensitive modifications that resulted in the same mutation frequency (unpublished results). Therefore, oxidative base modifications are calculated to be responsible for several percent of the overall mutagenicity of sunlight. Indeed, sequence analysis of mutations induced by simulated sunlight and UVA in cultured cells revealed a high proportion of T→G transversions, which are not observed with UVC (52). However, this type of base substitution is different from that known to result from 8-oxoG formation. The early saturation observed for the induction of Fpg-sensitive modifications by UVA and visible light (Figure 5) indicates that both the number of oxidative DNA modifications and their mutagenic (or carcinogenic) consequences can be underestimated when experiments with high doses and dose rates are extrapolated to the low doses and dose rates which may be relevant for many cells in the human skin.

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